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Original Article

Effects of interleukin 6 and tumor necrosis factor-α on the proliferation of porcine theca interna cells: Possible role of these cytokines in the pathogenesis of polycystic ovary syndrome

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ABSTRACT

Objective: We studied the effects of interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) on the proliferation of porcine theca interna (TI) cells and further elucidated the roles of IL-6 and TNF-α in the pathogenesis of polycystic ovary syndrome.
Materials and Methods: TI cells were treated with 10 pg/mL, 100 pg/mL, and 1000 pg/mL IL-6 or TNF-α. TI cell proliferation was then examined by carboxyfluorescein diacetate succinimidyl ester labeling and flow cytometry.
Results: Cell proliferation was not significantly different in TI cells cultured in medium alone (control) or in the presence of IL-6. At 72 hours of treatment, the mean fluorescence intensity was significantly lower in TI cells treated with 100 pg/mL and 1000 pg/mL TNF-α than in the control ($p < 0.05$).
Conclusion: TNF-α, but not IL-6, was able to promote TI cell proliferation. Our results suggest that TNF-α might play a role in hyperandrogenism, cortex thickness, and the increased ovary volume observed in polycystic ovaries.

Introduction

Polycystic ovary syndrome (PCOS) is a complex disorder of the nervous–endocrine–metabolic network. At present, its pathogenesis is unclear. Recently, many studies have reported that the expression of inflammatory factors, such as C-reactive protein (CRP), interleukin IL-6, and tumor necrosis factor-α (TNF-α) are higher in PCOS patients than in the normal population. Furthermore, the occurrence and development of PCOS have a close relationship with inflammatory factors [1–6].

The serum level of IL-6 in PCOS patients is higher than that in normal controls [2]. There are polymorphisms in the IL-6 gene promoter (−597G/A, −174G/C) of PCOS patients when compared to normal controls [7]. The IL-6 receptor consists of two heterodimeric subunits, IL6R-α (gp80) and IL6R-β (gp130). In gp130, the genotype frequency of Gly148Arg in PCOS patients is lower than in normal controls [8]. This study also found an association of IL-6 with the metabolism imbalance disease PCOS.

Yang et al [9] reported that the serum level of TNF-α in PCOS patients is higher than that in normal controls. Peral et al [10] reported that the methionine 196 arginine polymorphism in exon 6 of the TNF receptor 2 gene (TNFRSF1B) is associated with PCOS and hyperandrogenism. These findings suggest that inflammatory factors play a role in the pathogenesis of PCOS [10]. IL-6 and TNF-α levels in follicular fluid of PCOS patients are also higher than those in control women [11].

Recently, the inflammation theory has attracted significant attention. Inflammation is correlated with many diseases including atherosclerosis, diabetes mellitus, and obesity. Patients with these diseases have high levels of many inflammatory factors, indicating long-term and chronic subclinical inflammation (no acute inflammation symptoms). PCOS is often accompanied by obesity, diabetes

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mellitus, atherosclerosis, and coronary artery disease. However, it is unknown whether inflammation is correlated with the pathogenesis of PCOS.

PCOS may be an inflammatory disease, and the inflammation theory may explain its pathogenesis. Theca interna (TI) cells secret testosterone, and androstenedione. IL-6 and TNF-α are familiar to all. Therefore, in this study, we examined the effects IL-6 and TNF-α on TI cell proliferation to further explore the relationship between IL-6, TNF-α, and PCOS pathogenesis.

Materials and methods

Reagents and equipment

Porcine recombinant IL-6 and TNF-α were obtained from Prospec (Rehovot, Israel), Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) was purchased from Hyclone (Logan, UT, USA) and collagenase II was purchased from TBD (Tianjin, China). Hyaluronidase and trypsin were obtained from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was from Sijiqing (Hangzhou, China). A carboxyfluorescein diacetate succinimidyl ester (CFDA SE) cell proliferation and tracer detection kit were from Pik-day Biotechnology Research Institute (Lianyungang, China), and an aromatizing enzyme immunohistochemistry kit was purchased from Jingmei Biotech Co., Ltd (Shenzhen, China). Acetic acid (36%) was obtained from Shanghai Chemical Reagent Company (Shanghai, China). The fluorescence microscope (TE2000-U) was purchased from Nikon Eclipse (Tokyo, Japan). The FACSCalibur flow cytometer was purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

Isolation and culture of primary porcine TI cells

Fresh porcine ovaries were acquired from an abattoir and placed in ice-cold two-antibiotic physiological saline (containing 800,000 IU penicillin and 800,000 IU streptomycin per 500 mL). The ovaries were kept on ice and transported to the laboratory within 2 hours of slaughter. The ovaries were washed repeatedly with normal physiological saline. Healthy follicles with a diameter of 4–6 mm were carefully cut in half with microdissection scissors under a dissecting microscope to allow the fluid to flow out. Granulosa cells (GCs) attached to the surface were scraped off with a curette. The hat-shaped folliculi interna thecas were separated with micro-tweezers and washed several times with physiological saline. The thecas were shredded and then digested with an enzyme solution (0.25% collagenase II, 0.05% hyaluronidase, and 0.05% trypsin) in a 37°C incubator with 5% CO_2 for 20–50 minutes. The samples were then filtered through a 100 mesh stainless steel filter. The filtered cell suspension was centrifuged for 5 minutes at 682 ×g and then washed with normal physiological saline.

The obtained TI cells were resuspended in DMEM/F12 containing 1% two-antibiotic and 10% FBS. Trypan blue staining showed >70% viability.

Assessment of TI cell purity

TI cells are often contaminated with ovarian GCs. Aromatizing enzyme converts testosterone into estradiol and androstenedione into estrone, which is expressed in GCs but not TI cells. Therefore, we tested for the presence of aromatizing enzyme to determine the purity of TI cells by immunohistochemistry.

TI cells were cultured in six-well plates containing coverslips. The cell suspension was diluted to 1×10^5 cells/mL by addition of 3 mL DMEM/F12 containing 10% FBS. The cells were incubated at 37°C with 5% CO_2. The culture medium was changed after 48 hours, and the purity of the cells was determined according to the immunohistochemistry kit instructions. GCs were stained, while TI cells were unstained. The results showed < 10% GCs.

CFDA SE labeling of cells and stimulation with IL-6 and TNF-α

TI cells were labeled according to the instructions of the CFDA SE detection kit. The labeled TI cells were diluted to 1×10^5 cells/mL by addition of 1 mL DMEM/F12 and seeded in 24-well plates. IL-6 or TNF-α were then added to the cultures at final concentrations of 0 pg/mL (control), 10 pg/mL, 100 pg/mL, or 1000 pg/mL in triplicate wells. The cells were incubated at 37°C with 5% CO_2. The media were refreshed after 48 hours, and flow cytometry was performed after 72 hours.

CFDA SE (formula C29H19NO11; molecular weight 557.47) is a dye that can penetrate cell membranes, it is catalyzed into CFSE by the esterase activity of the cells. CFSE binds spontaneously and irreversibly to acellular protein lysine residues or other available amine groups, marking the proteins. Full labeling is achieved at about 24 hours after addition of the fluorescent probe. Because the fluorescence of cells labeled with CFDA SE is uniform and stable, the fluorescence of progeny cells will be halved with every division. Therefore, flow cytometry can be used to detect these varying degrees of fluorescence in CFDA SE-labeled cells to detect up to eight cell divisions.

Flow cytometric detection of TI cell proliferation

After collection by trypsinization, each group of cells was washed once with PBS and then diluted with PBS to an appropriate concentration for flow cytometric analysis. Ten thousand cells were analyzed by the flow cytometer. FlowJo (Tree Star, Inc) software was used to analyze the mean fluorescence intensity (MFI) of each group of cells.

Statistical analysis

Data were analyzed with SPSS 16.0 (SPSS Inc., Chicago, IL) and are expressed as the mean ± standard deviation. Comparisons among multiple groups were analyzed by one-way analysis of variance. A p value of < 0.05 was considered statistically significant.

Results

Effects of IL-6 on TI cell proliferation

The MFI of the three experimental groups treated with 10 pg/mL, 100 pg/mL, or 100 pg/mL IL-6 were compared with the untreated control group at 72 hours. The results are shown in Table 1. Cell proliferation was not significantly different in TI cells treated with or without IL-6.

Table 1
Mean fluorescence intensity (MFI) of theca interna cells treated with various concentrations of interleukin-6 ($n = 9$; 72 hours of treatment).

Group (pg/mL)	MFI	p
0	66.9 ± 14.8	
10	68.6 ± 13.4	> 0.05
100	65.8 ± 6.7	> 0.05
1000	63.7 ± 8.81	> 0.05

Effects of TNF-α on TI cell proliferation

The MFI of cells treated with 100 pg/mL or 1000 pg/mL TNF-α at 72 hours was significantly lower than that of untreated cells (53.2 ± 11.59 vs. 66.87 ± 14.8, $p = 0.013$; 47.55 ± 7.31 vs. 66.87 ± 14.8, $p = 0.001$), whereas 10 pg/mL TNF-α had no significant effect on the MFI (60.2 ± 9.2 vs 66.87 ± 14.8, $p = 0.21$; Figures 1–5 and Table 2).

Discussion

The ovary is composed of the cortex and medulla. The outer peripheral portion of the cortex in the ovary is thick with follicles at various development stages, the corpus luteum, as well as atresic and degenerative follicles. The medulla is located in the center of the ovary, and is mainly composed of loose connective tissue. In secondary follicles, the theca constantly thickens with the growth of the follicles, forming the inner and outer membranes. The outer membrane is mainly composed of dense connective tissue, a small number of spindle cells, blood vessels, and smooth muscle. The inner membrane consists of loose connective tissue and abundant vessels, which contains a large number of theca cells. At this time, the theca cells have a large volume and nucleus, prominent nucleoli, and the cytoplasm is enriched with smooth endoplasmic reticulum, tubular cristae of mitochondria, and lipid droplets with typical characteristics of steroid synthesis.

The cholesterol from plasma is converted to pregnenolone in the mitochondria of follicular GCs and theca cells. Pregnenolone is converted to androstenedione and testosterone by 17α-hydroxylase and 17, 20-lyase, in the smooth-surfaced endoplasmic reticulum of TI cells. Pregnenolone can be converted to progesterone, and then progesterone is converted into androstenedione and testosterone by 3β-hydroxysteroid dehydrogenase in TI cells. Therefore, TI cells produce and secrete androgen.

Typical pathological changes of PCOS include a significant enlargement (2–5 times) and gray appearance of the ovaries with a smooth surface and thickened cortex (up to a 33% increase) that contains a large number of cystic follicles with unequal sizes as well as colorless and transparent cyst fluid. In the cystic follicles, microscopic examination showed that the GCs which synthesize estrogen decrease in layers and quantities. Part follicles are atretic, GCs undergo apoptosis and disappear. Thecal cells in follicles, which synthesize androgen, proliferate significantly.

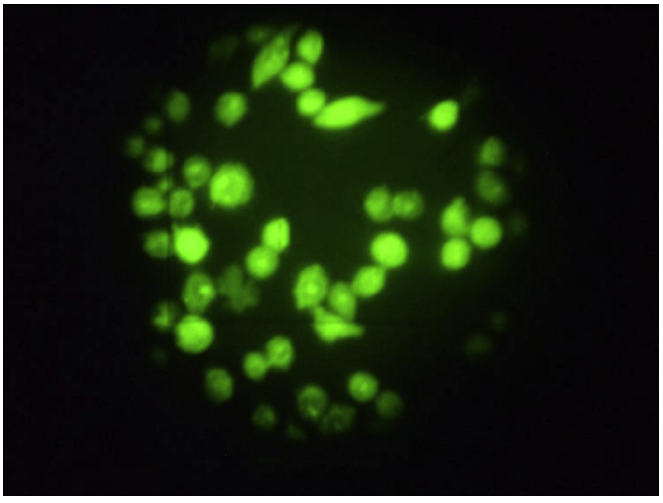

Figure 1. Fluorescence of theca interna cells at 72 hours (control).

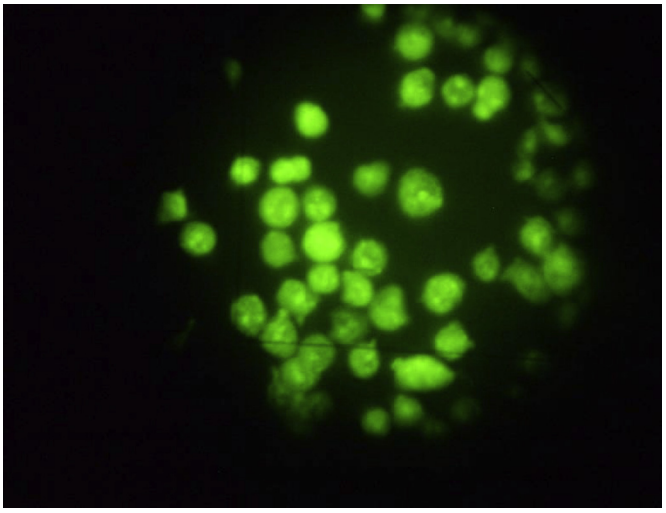

Figure 2. Fluorescence of theca interna cells at 72 hours (tumor necrosis factor-α 10 pg/mL).

Ultrastructural observations indicate that GCs have a large amount of rough endoplasmic reticulum, multiple ribosomes, well-developed Golgi, and many mitochondria, which remind the decreased levels of estrogen synthesis. Thecal cells also have a large amount of smooth endoplasmic reticulum, as well as small lipid droplets and tubular mitochondria, indicating active androgen synthesis [12].

In 1975, Carswell et al [13] found a factor that could destroy tumor cells and cause hemorrhaging and necrosis in tumor tissue *in vivo*. It was named tumor necrosis factor by Old [14]. TNF-α has a broad range of sources including various immune cells, endothelial cells, fibroblasts, epithelial cells, and smooth muscle cells with activated monocytes/macrophages cells as the main source. TNF-α also has a wide range of biological activities. It is anti-infective and can cause fever, destroys or inhibits tumor cells, enhances the phagocytic capacity of neutrophils [15], strengthens the function of antibody-dependent cell mediated cytotoxicity, induces acute-phase protein synthesis in liver cells, inhibits virus replication, and plays an important role in the regulation of sugar and fat metabolism.

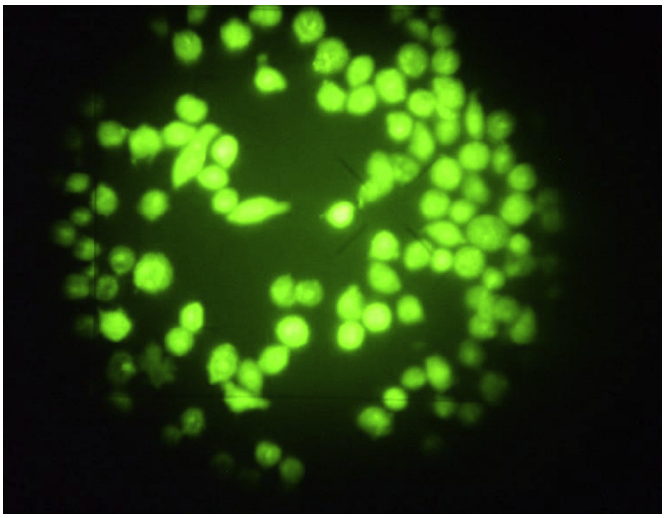

Figure 3. Fluorescence of theca interna cells at 72 hours (tumor necrosis factor-α 100 pg/mL).

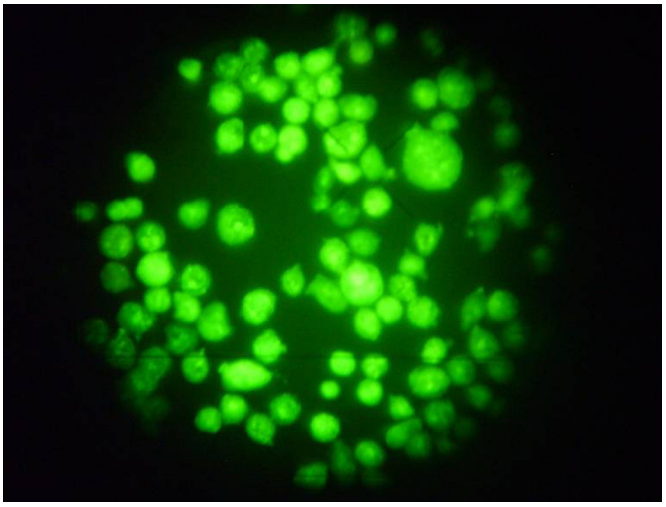

Figure 4. Fluorescence of theca interna cells at 72 hours (tumor necrosis factor-α 1000 pg/mL).

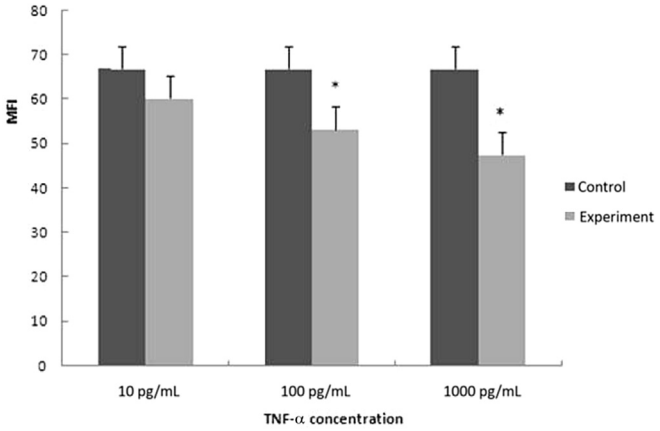

Figure 5. Effect of tumor necrosis factor (TNF)-α on the proliferation of theca interna cells at 72 hours. $n = 9$. * $p < 0.05$ vs. control. MFI = mean fluorescence intensity; TNF-α = tumor necrosis factor-α.

Table 2
Mean fluorescence intensity (MFI) of theca interna cells treated with various concentrations of tumor necrosis factor-α ($n = 9$; 72 hours of treatment).

Group (pg/mL)	MFI	*p*
0	66.9 ± 14.8	
10	60.2 ± 9.2	> 0.05
100	53.2 ± 11.6	< 0.05
1000	47.6 ± 7.3	< 0.05

TNF receptors are present on the surface of almost all types of normal cells, as well as a variety of tumor cells. The mechanism of signal transduction followed by TNF-α binding to its corresponding receptor is not yet clear. Present studies have shown that TNF-α has three different signaling pathways that generate distinct effects, including apoptosis, nuclear factor-κB, and c-Jun N-terminal kinase signaling pathways [16].

It has been reported that TNF-α has a role in the promotion of hyperandrogenism [17]. Our study revealed that high doses of TNF-α stimulated the proliferation of TI cells, indicating that TNF-α has a role in the thickness of the cortex, the increased ovary volume, and even the hyperplasia of follicle numbers, the tunica albuginea, and ovarian medulla observed in PCOS ovaries. The mechanism of TNF-α stimulation of cell proliferation is related to activation of JNK and other signal transduction pathways [18]. TNF-α can also induce insulin resistance [2], followed by the occurrence of PCOS.

The human IL-6 gene is located in the short arm of chromosome 7. It is about 5 kb including five exons and four introns. Mature IL-6 contains 184 amino acid residues with a molecular weight was 26 kD. T cells, B cells, monocytes, fibrocytes and fat cells produce IL-6. IL-6 accelerates B cell proliferation, differentiation, and secretion of antibodies. IL-6 has an anticancer effect, IL-6 promotes the anti-tumor activity of natural killer cells and cytotoxic T lymphocytes directly or indirectly. IL-6 also promotes acute phase protein levels in the liver and inflammatory reactions, and contributes to the growth of hemocytes.

In our study, we showed that IL-6 did not directly promote the proliferation of TI cells. Therefore, it is unclear how IL-6 participates in the pathogenesis of PCOS. It is well known that the activity of androgen is influenced by several factors such as androgen receptor (AR). It has been reported that IL-6 upregulates AR expression in prostate cancer [19]. Similarly, IL-6 might also enhance androgen activity by upregulation of AR expression in the ovary, leading to PCOS. In addition, IL-6 might cause insulin resistance first, followed by PCOS [2].

Further studies are needed to demonstrate a connection between inflammatory factors and PCOS pathogenesis. By verifying the involvement of inflammatory factors in the pathogenesis of PCOS, new methods may be developed for PCOS treatment, such as blocking therapeutic targets including inflammatory factors and signaling pathways.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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